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Prognostic and predictive significance of p53, EGFr, Ki-67 in larynx preservation treatment

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ABSTRACT

Background: The optimal management of advanced laryngeal and hypopharyngeal cancers (L&HC) must involve consideration of both survival and functional effect of the given treatment approach. Despite over two decades of investigations of several treatment options, including surgery, radiotherapy, chemotherapy or some combinations thereof, little consensus exists as to which treatment offers the best survival, together with functional speech and swallowing.

Aim: To determine predictive and prognostic value of p53, EGFr, Ki-67 in patients with advanced laryngeal and hypopharyngeal cancer, treated with larynx preservation intent.

Materials and methods: Thirty-three patients received 2–3 cycles of induction chemotherapy (ICHT) consisting of cisplatin and fluoruracil and underwent subsequent radical radiotherapy. Immunohistochemical analyzes of p53, EGFr and Ki-67 were performed.

Results: Response to ICHT was obtained in 24 patients (75%). Better response to ICHT was correlated only with EGFr expression ($p = 0.04$, RR = 1.91). The 5-year loco-regional control (LRC) and disease-specific survival (DSS) rates were 48% and 57%, respectively. The 5-year larynx preservation rate was 68% in responders to ICHT compared to 21% in non-responders ($p = 0.02$). It was also higher in patients without EGFr expression (but not significantly, $p = 0.43$).

Conclusion: Lack of EGFr expression is a favorable predictive factor for response to ICHT. Neither p53 nor Ki-67 have predictive and prognostic value in larynx preservation treatment.

1. Background

The optimal management of advanced squamous cell laryngeal and hypopharyngeal cancers (L&HC) must involve

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consideration of both survival and functional effect of the given treatment approach. Despite over two decades of investigations of several treatment options, including surgery, radiotherapy, chemotherapy or some combinations thereof, little consensus exists as to which treatment offers the best survival, together with functional speech and swallowing.[1] In the 1990s, a standard treatment option for patients with advanced L&HC who wanted to preserve their voice and swallowing was induction chemotherapy (ICHT) followed by radiotherapy.[2,3] The new standard of care was established after publishing the results of RTOG 91-11 trial which showed significant increase in larynx preservation rate after radiotherapy with concurrent cisplatin compared to ICHT followed by radiotherapy or radiotherapy alone.[4,5]

In the above studies, ICHT was used as a predictor of radiosensitivity. Selection criteria for patients who may benefit from combining chemotherapy with radiotherapy remain an unresolved problem. Researchers are looking for new factors predicting chemosensitivity, radiosensitivity and finally, chances of larynx preservation. Studies on a number of molecular markers have been undertaken. One of these markers is p53 protein (product of the *p*53 suppressor gene). Mutation of *p*53 is the most common genetic alteration identified thus far in human cancers, and it plays an important role in cell proliferation control.[6] Its role as a predictor of chemosensitivity and radiosensitivity remains contradictory.[7–9] Another potential marker is epidermal growth factor receptor (EGFr). Its expression, which exists in 80–100% of advanced head and neck cancers, is connected with higher aggressiveness of cancers.[10–14] Inhibition of EGFr by monoclonal antibody brakes activation of tyrosine kinase which in turn leads to impairment or even stopping of cell cycle. Combination of antibody against EGFr with cisplatin or doxorubicin causes stronger toxic effect than each of the compounds separately.[15,16] It is also proven that the blockade of EGFr by antibody increases radiosensitivity of squamous cell cancer of the head and neck.[17,18] Cell cycle proliferation plays a very important role in head and neck cancer. One of the most often measured markers of proliferation is Ki-67 antigen. Squamous cell cancer of the head and neck generally shows increased expression of this marker, but its predictive and prognostic value remains unclear, especially in patients treated with ICHT.[19–23]

2. Aim

The aim of this retrospective study is to determine predictive and prognostic value of p53, EGFr, Ki-67 in patients with advanced L&HC treated with larynx preservation intent.

3. Materials and methods

3.1. *Patients and treatment*

The data from 32 patients with advanced L&HC treated between January 1988 and December 1997 were analyzed retrospectively. All patients were unfit for surgery because of advancement of the primary tumor or co-existing morbidities. All patients received 2–3 cycles of ICHT: 2–16 patients (50%), 3–16 patients (50%). Induction chemotherapy consisted of cisplatin (100 mg/m^2 as a 1-h infusion on day 1) and fluorouracil (1000 mg/m^2 as a 24-h infusion on days 1–5). The cycle was repeated every 4 weeks. After finishing ICHT, all patients underwent subsequent radical radiotherapy. All patients were irradiated using megavoltage unit. Total tumor dose was 62–72 Gy (median 68 Gy), fraction dose was 2.0 Gy delivered 5 times a week, overall treatment time was 40–51 days (median 43 days).

3.2. *Histologic and immunohistochemical assessment*

Histologic and immunohistochemical analyses were performed at the Pathology Department of the Oncology Center in Cracow, basing on archival paraffin blocks. All immunohistochemical assays were performed using the unstained 5-μm tissue sections. The sections were mounted on silanized slides (Menzel-Glasser), baked for 1 h at 650 °C and stored at room temperature until used. Sections were de-paraffined in xylene and rehydrated in alcohol. Endogenous peroxidase was blocked with 0.3% H_2O_2 followed by two washes in de-ionized water. For antigen retrieval, slides were treated with citrate buffer (pH 6.0) in a microwave oven (560 W), cooled, washed in TRIS buffer and then incubated with normal swine serum (DAKO X0901) 1:10. Slides for Ki-67 antigen visualization were additionally treated with trypsin (DAKO S2012) 0.05% (5 min). Primary antibodies were incubated overnight at 40 °C in a humidity chamber (dilutions in table below), followed by 15 min of incubation with biotinylated secondary antibody and 15 min with streptavidin–HRP complex (LSAB2/HRP kit, DAKO S3000) 10 min. After washing in TRIS and water, counterstaining and differentiation with 3% HCl in alcohol, the slides were washed in de-ionized water, dehydrated in graded concentration of alcohol, cleared in xylene and covered. Tumors were considered EGFR positive only if clear brown membrane staining was noticed. Tumor cells were considered positive for Ki-67 only when there was clear nuclear staining. The number of positively staining tumor cells per 500 tumor cells was counted and the percentage of positively staining cells was assessed. Evaluation of the p53 reactions was based on staining intensity and estimation of the percentage of positive tumor cell nuclei. Results of histologic and immunohistochemical assessment together with patients' characteristics are shown in Table 1.

3.3. *Statistical methods*

The criteria of assessment of the treatment results included:

1. Overall response to induction chemotherapy defined as complete (CR) or partial (PR) regression of the primary tumor and metastatic lymph nodes, according to WHO criteria.[24]
2. Loco-regional control (LRC).
3. Disease-specific survival (DSS).

Analysis of dependence between assessed factors and univariate analysis of influence of assessed factors on the chance of achieving response to ICHT were performed using

Table 1 – Patients' characteristics.

Factor	No. of pts.	%
Sex		
Male	30	94
Female	2	6
Age (median 57 years, range 43–73)		
<57	15	47
≥57	17	53
KPS		
≤60	7	22
>60	25	78
Primary site		
Larynx	30	88
Hypopharynx	2	12
T-stage		
T3	10	31
T4	22	69
N-stage		
N0	17	53
N1–3	15	47
Clinical stage		
III	7	22
IV	25	78
Histologic grading		
G1 + G2	16	50
G3	16	50
EGFr status		
No expression	21	66
Expression	11	34
p53 status		
No expression	13	41
Expression	19	59
Ki-67 status (median 59%, range 13–97%)		
<59%	18	56
≥59%	14	44

Abbreviations: no. of pts. – number of patients, KPS – Karnofsky performance status, EGFr – epidermal growth factor receptor.

Table 2 – Response rates to ICHT in relation to analyzed factors (Chi-square test of independence).

Factor	No. of pts.	Response to ICHT (%)	*p*
Sex			
Male	30	82	
Female	2	50	0.49
Age			
<57	15	72	
≥57	17	88	0.17
KPS			
≤60	7	59	
>60	25	79	0.18
T-stage			
T3	10	83	
T4	22	76	0.51
N-stage			
N0	17	81	
N1–3	15	82	0.92
Clinical stage			
III	7	82	
IV	25	29	0.66
Histologic grading			
G1 + G2	16	71	
G3	16	84	0.26
EGFr status			
No expression	21	94	
Expression	11	62	0.03
p53 status			
No expression	13	87	
Expression	19	83	0.89
Ki-67 status			
<59%	18	77	
≥59%	14	86	0.74

Abbreviations: no. of pts. – number of patients, KPS – Karnofsky performance status, ICHT – induction chemotherapy, EGFr – epidermal growth factor receptor.

Chi-square test of independence. Multivariate analysis was performed using the logistic model. Five-year LRC and DSS rates were assessed using the Kaplan–Meier method. Differences between groups were compared with log-rank test. The multivariate analysis was performed using the Cox proportional hazards regression model. All the results with p value ≤0.05 were considered statistically significant. Statistical analyses were performed using STATISTICA v.6.0 software by StatSoft.

4. Results

4.1. *Response to induction chemotherapy*

Response to ICHT was obtained in 24 patients (75%). Response rates to ICHT in relation to the analyzed factors are shown in Table 2. Significantly higher response was achieved in patients without EGFr expression. It was confirmed in multivariate analysis (logistic model). Patients having tumors without expression of EGFr present approximately two times higher chance of responding to ICHT ($p = 0.04$, RR = 1.91).

4.2. *Loco-regional control and disease-specific survival*

The follow-up time, i.e. time between the date of starting treatment and the date of patient's death or the last follow-up, was 10–102 months (mean 43, median 42). During the follow-up period 21 patients died – 13 because of primary cancer, 8 because of other causes. The 5-year LRC and DSS rates assessed using the Kaplan–Meier method were 48% and 57%, respectively. The differences in 5-year LRC and DSS rates according to the analyzed factors compared with log-rank test are shown in Table 3. Statistically significant unfavorable prognostic factors for LRC were: KPS ≤ 60, lack of response to ICHT, and EGFr expression. Statistically significant unfavorable prognostic factors for DSS were: KPS ≤ 60, lack of response to ICHT and lymph nodes involvement. Multivariate analysis using the Cox proportional hazards regression model, shows unfavorable influence of lack of response to ICHT on LRC ($p = 0.01$; RR = 5.48) as well as on DSS ($p = 0.03$; RR = 4.12).

Table 3 – The 5-year LRC and DSS according to the analyzed factors (log-rank test).

Factor	No. of pts.	LRC (%)	p	DSS (%)	p
Sex					
Male	30	51		55	
Female	2	39	0.85	51	0.62
Age					
<57	15	43		56	
≥57	17	59	0.07	71	0.07
KPS					
≤60	7	13		15	
>60	25	51	0.04	59	0.02
T-stage					
T3	10	43		47	
T4	22	55	0.44	64	0.75
N-stage					
N0	17	53		67	
N1–3	15	41	0.20	42	0.04
Clinical stage					
III	7	43		51	
IV	25	52	0.74	59	0.73
Response to ICHT					
PR + CR	24	55		61	
No response	8	17	0.03	33	0.02
Histological grading					
G1 + G2	16	46		45	
G3	16	47	0.96	57	0.98
EGFr status					
No expression	21	44		55	
Expression	11	21	0.04	36	0.24
p53 status					
No expression	13	53		58	
Expression	19	22	0.28	36	0.20
Ki-67 status					
<59%	18	58		69	
≥59%	14	37	0.33	37	052

Abbreviations: no. of pts. – number of patients, LRC – loco-regional control, DSS – disease-specific survival, KPS – Karnofsky performance status, ICHT – induction chemotherapy, PR + CR – partial response + complete response, EGFr – epidermal growth factor receptor.

Regional lymph nodes involvement was unfavorable prognostic factor influencing DSS ($p = 0.01$).

4.3. *Larynx preservation and treatment failures*

Larynx preservation rate after 5 years was 68% in patients who respond to ICHT, compared to only 21% in non-responders ($p = 0.02$). It was also higher in patients without EGFr expression (but not significant, $p = 0.43$). Treatment failures were observed in 19 patients (59%). The most common causes of failure were: local failure or/and recurrence – 11 patients (35%), regional failure or/and recurrence – 10 patients (31%). Of these patients, 6 (6/19 – 33%) underwent salvage surgery. Distant metastases developed in 2 patients (6%) – both in the lungs.

5. Discussion

In this retrospective study we analyzed a homogenous group of 32 patients with advanced L&H cancer. All patients received the same type of ICHT. Subsequent treatment varied depending on response to ICHT and performance status.

Overall response to ICHT (75%) is similar to cited in the literature.[2–4,25] Lack of EGFr expression is the only independent factor predicting response to ICHT in multivariate analysis. There is no correlation of EGFr expression with any other of the examined factors. There are no data in the literature concerning correlation between p53, EGFr and Ki-67 expression and response to ICHT in patients treated with larynx preservation intent. To our knowledge, only one retrospective study has been published on EGFr in larynx preservation treatment involving ICHT.[25] Unfortunately, the authors did not mention the influence of EGFr on response to ICHT. Some researchers suggest that EGFr concentration of more than 100 fmol/mg of membrane protein predicts better response to chemotherapy in head and neck cancer patients.[10] Others did not show any correlation between EGFr expression and the results of chemotherapy.[11,14] Studies undertaken by Veterans Affairs Laryngeal Cancer Study Group (VALCSG) showed significant increase in response rates to ICHT among patients with tumors overexpressing p53 (89% compared to 74%, respectively).[7,8] On the contrary, Etienne et al.[14] observed lower p53 expression in responders ($p = 0.06$). On the other hand, a study from Memorial Sloan-Kettering Cancer Center revealed no influence of p53 on response to ICHT.[9] We observe decreased response rates to ICHT in patients with Ki-67 expression in less than 59% of cells. The difference reaches 10% but is statistically insignificant. After decades of explorations, predictive and prognostic role of Ki-67 remains unclear. There are no published data concerning its influence on response to ICHT, especially in larynx preservation treatment. It may be explained by the fact that Ki-67 is considered to be a predictor of radiosensitivity rather than of chemosensitivity. Even though the data are contradictory,[19–22] some authors suggest possible misinterpretation of Ki-67 expression. Cell proliferation varies in different parts of tumor, and Ki-67 proves that a cell is in the proliferation phase rather than indicate how fast that proliferation really is.[26,27]

Larynx preservation rate among our patients reaches 50% after 5 years of follow-up. The lack of EGFr expression is the only statistically significant predicting factor for larynx preservation. Our results are comparable to those published in the literature.[2,3] Meta-analysis of randomized trial in head and neck cancer showed only slightly better results.[28] There is only one retrospective study showing that among patients receiving ICHT with subsequent radiotherapy, those with EGFr level equal or higher than 100 fmol/mg had significantly worse disease-free survival (22% compared with 53%).[25] Some other show increased rate of loco-regional recurrence rate correlated with overexpression of EGFr.[11,12,29] Reviewing the literature we found that majority of researchers observe unfavorable influence of p53 on larynx preservation rates[9,13] or relapse-free survival.[12] Only study done by VALCSG showed increased larynx preservation rates in those who overexpress p53 (74% compared to 53%, $p = 0.03$).[7] The role of Ki-67 remains unclear.

Some suggest that it has no impact on larynx preservation as a single factor,[20,21] others state that its lower expression together with overexpression of p53 decrease local control rate ($p = 0.002$).[22] On the other hand, Lavertu et al.[21] showed that low Ki-67 expression together with lower p53 expression increase relapse-free survival rates. These different results may be explained in the same way as mentioned above. It should be also considered that the above-cited authors used different cut-off levels to define low or high expression of the examined factors.

Until now, the results from studies on biomarkers have had no impact on clinical practice, possibly because there are no data from large trials that specifically estimate L&HC. The published data are not homogenous, different methods of assessment are used which probably could account for the conflicting data. Among recently published data are those from trials concerning larynx preservation treatment with new ICHT regimens including taxanes. The results are encouraging; low toxicity is connected with high rates of larynx preservation.[30,31] Results have been also presented concerning targeted therapy with antibody against EGFr combined with radiotheray.[32] Although that trial was not designed for organ preservation, the results are promising. Introduction of taxanes and targeted therapy into larynx preservation treatment, together with standardization of biomarkers assessment, is a challenging way in the treatment of patients with advanced L&HC.[33]

6. Conclusion

Lack of EGFr expression is a favorable predictive factor for response to ICHT. Larynx preservation rate is higher in those who respond to ICHT and have no expression of EGFr.

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